Laboratory tests for diagnosing and monitoring canine leishmaniasis

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Abstract: Although several reviews on canine leishmaniasis have been published, none thoroughly described clinicopathologic abnormalities and their clinical usefulness. The aim of this review was to provide information concerning current diagnostic tests relevant for clinical pathologists and from a practical perspective. Specifically, in canine leishmaniasis, nonregenerative normocytic normochromic anemia, thrombocytopenia, or leukogram changes may be present. Clinical chemistry and urinalysis may indicate renal dysfunction (azotemia, decreased urine specific gravity, proteinuria) and an inflammatory/immune response (increased acute phase proteins [APP] or 2 - and/or -globulins). Although a potential gammopathy is usually polyclonal, it may also appear oligo- or monoclonal, especially in dogs coinfected by other vector-borne pathogens. When lesions are accessible to fine-needle aspiration (lymphoadenomegaly, nodular lesions, joint swelling), cytology is strongly advised, as the presence of Leishmania amastigotes in a pattern of pyogranulomatous inflammation or lymphoplasmacytic hyperplasia is diagnostic. If the cytologic pattern is inconclusive, the parasite should be identified by histology/immunohistochemistry or PCR on surgical biopsies. Alternatively, cytology and PCR may be performed on bone marrow samples where amastigotes, along with erythroid hypoplasia, myeloid hyperplasia, plasmacytosis, or secondary dysmyelopoiesis can be observed. Dogs with overt leishmaniasis generally have high antibody titers, while low titers predominate in immunologically resistant infected dogs or in exposed dogs with no parasite confirmation. Quantitative serology is recommended in clinically suspect dogs as high-titer antibodies titers may confirm the clinical diagnosis. In confirmed and treated dogs, renal function and inflammatory/immune response variables should be periodically monitored.

DOI: https://doi.org/10.1111/vcp.12413

LABORATORY TESTS FOR DIAGNOSING AND MONITORING CANINE LEISHMANIASIS

Running header: laboratory diagnosis of leishmaniasis

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Abstract

Despite several reviews on canine leishmaniasis, none throughly described laboratory abnormalities and diagnostic tests in light of their clinical usefulness. Aim was to summarize this information from a practical perspective. Canine leishmaniasis often leads to profound clinicopathological abnormalities. CBC reveals non-regenerative normocytic normochromic anemia and thrombocytopenia, but other types of anemia or leukogram changes are also observed. Clinical chemistry and urinalysis frequently show abnormalities suggestive of renal dysfunction (increased creatinine and urea, decreased urine specific gravity, proteinuria) and inflammatory/immune activation (increased acute phase proteins or α_2- and/or γ-globulins). The gammopathy is typically polyclonal but can also be oligo- or monoclonal. When lesions accessible to fine needle aspiration or other sampling techniques (lymhpoadenomegaly, nodular or ulcerated skin lesions, joint swelling) are present, cytology is strongly advised; the disease is confirmed if *Leishmania* amastigotes are identified and pyogranulmatous inflammation or lymphoplasmocytic hyperplasia is present. If results are inconclusive, the parasite should be identified through histology/immunohistochemistry or PCR on surgical biopsies. Alternatively, cytology and PCR may be performed on bone marrow, where amastigotes and erythroid hypoplasia, myeloid hyperplasia, plasmocytosis, or secondary dysmyelopoiesis can be observed. Dogs with overt disease generally have high antibody titers at ELISA or immunofluorescence. Low antibody titers may be found in infected dogs that are immunologically resistant, or in exposed dogs (dogs not harboring the parasite). If leishmaniasis is suspected but cytological evidence of the parasite is negative, serology is recommended for confirmation. In treated dogs, renal function and the magnitude of the inflammatory/immune reaction should be periodically monitored.

Keywords: Dog; Leishmania infantum; clinical usefulness; diagnosis; follow-up

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Leishmaniasis is a frequent infectious disease in dogs living in endemic areas, is associated with important morbidity and, although appropriate treatment, it can cause death. Despite several reviews have been published so far, none has fully described the diagnostic role of laboratory findings that are observed in dogs with leishmaniasis and of available tests. Therefore, the aim of the present review was to provide information concerning laboratory abnormalities and current diagnostic tests, from a practical perspective.

Etiology and pathogenesis of canine leishmaniasis

Canine leishmaniasis is a disease caused by the protozoan parasite *Leishmania infantum* (Kinetoplastida: Trypanosomatidae), or its New World synonym *Leishmania chagasi*.[1] Although non-vectorial transmission is occasionally reported (e.g. by transplacental, transfusional or venereal route),[2-4] it is assumed that canine leishmaniasis is mainly acquired by the bite of infected phlebotomine sand flies (Diptera: Psychodidae). Therefore, the geographical distribution and prevalence of the disease largely depends on the presence and abundance of competent vector species, including members of the *Phlebotomus* (*Larroussius*) subgenus in the Old World (Mediterranean Basin and Central Asia) and *Luztomyia longipalpis* and *L. evansi* in the New World (from Mexico through Argentina).[5] Blood-sucking females ingest the intracellular, non-flagellated form of *Leishmania* (amastigote) during the blood meal on infected hosts. After multiplication, flagellated forms (promastigote) and transform into non-replicative, infectious metacyclic forms that are inoculated into the host's dermis when the fly takes another blood meal. Parasites are phagocytosed by local and recruited host cells, and within phagolysosomes of resident macrophages promastigotes transform and replicate as amastigotes.[6]

The pathogenesis of the disease starts with the ingestion of recently inoculated promastigotes by neutrophils that are unable to kill the protozoa but activate dermal macrophages and other mechanisms of innate immunity (e.g. acute phase response). Macrophages initially activate their antimicrobial defenses, which are based on the release of enzymes and on the production of free

radicals within the phagolysosome. *Leishmania* organisms are able to interfere with the oxidative activity of the phagocytes[7,8] and therefore amastigotes can survive and actively replicate in macrophages, leading to cell destruction and infecting progressively more and more phagocytes.

In longitudinal field studies employing naïve dogs, it has been demonstrated that *Leishmania* can be detected by PCR in bone marrow samples starting about 6 months from natural exposure to phlebotomine vectors.[9] Once bone marrow has been colonized it is generally accepted that the dog is persistently infected. However, the same studies showed that dogs being PCR positive in bone marrow may become negative in the following months without any treatment; it is unknown whether the parasite density fluctuates below the threshold limit of the test, the infection persists in organs other than bone marrow, or the host defenses successfully eradicate the infection.[9] Despite dogs can mount an antibody response after the first contact with the parasites, resistance or susceptibility to progressive infection (leading to clinical disease) depend on the balance between Th1 (cell-mediated) and Th2 (humoral) immune responses of the host: those with prevailing Th2 response are more likely to have uncontrolled dissemination of parasites to all body tissues and to show lesions and clinical signs of leishmaniasis.[10-13]

Of note, depending on the host-parasite interactions, the simple detection of circulating antibodies does not necessarily imply that the dog is actually infected and detection of parasites in tissues does not suggest that the infected dog is actually sick. Therefore, the two existing guidelines that deal with the diagnosis and staging of canine leishmaniasis, recently released by the Leishvet group[14] and the Canine Leishmaniasis Working Group (CLWG),[15] classify dogs as exposed, infected or sick based on a combination of clinical findings and clinicopathological tests or tests for etiological diagnosis. Specifically, both guidelines include two main categories of dogs:

- Infected dogs: dogs clinically unremarkable, without abnormalities in hematology, clinical chemistry and urinalysis, that test positive to PCR or cytology in tissues such as bone marrow, lymph node, spleen, skin or peripheral blood;

- Sick dogs: infected dogs that have clinical signs or clinicopathological changes consistent with leishmaniasis.

The classification of the CLWG[15] includes two additional categories of dogs at the extremes of the spectrum:

- Exposed dogs: dogs clinically unremarkable with positive serology, in which PCR or cytology fail to demonstrate the presence of the parasite;
- Severely sick dogs: sick dogs that do not respond to treatments and/or are affected by concurrent diseases.

The Leishvet group classifies sick dogs in four stages according to the severity of clinical signs and magnitude of antileishmanial antibody titers.[14]

Clinical signs of canine leishmaniasis

The interpretation of clinicopathological, serological and molecular tests should be done in light of history, signalment and clinical presentation. History should focus on the risk of exposure to phlebotomine vectors. Signalment should focus on gender, breed and age; clinically overt leishmaniasis is more frequent in male dogs, Boxer and German shepherd, and in those younger than 3 years or older than 8 years, [16,17] although recent reports suggests that any dog above 2 years is at risk.[18]

The spectrum of clinical presentations is wide and ranges from infections characterized by the absence of obvious clinical findings but detectable laboratory abnormalities, to overt clinical infections characterized by the presence of clinical and laboratory abnormalities that require or not hospitalization. However, some signs are more frequent than others. The most typical clinical findings include:[14,15,19-22] generalized lymphoadenomegaly, cutaneous lesions, pale mucous membranes, weight loss or cachexia, polyuria/polydipsia, epistaxis, onicogriphosis. Ocular lesions,[15,23,24] lameness, lethargy and fever are also quite common.[25,26] In a longitudinal study,[27] it was shown that lymph node enlargement appears first (approximately 1 year after exposure to sand

flies), followed by cutaneous lesions (after 18 months) and ocular signs (after 22 months). Furthermore, atypical forms have been described, especially in highly endemic areas, including gastrointestinal, neurological, musculoskeletal, cardiopulmonary, lower urinary tract or genital tract signs.[28-36]

Laboratory abnormalities that may support or confirm leishmaniasis

In addition to clinical findings, laboratory abnormalities detectable by routine hematology, clinical chemistry or urinalysis may further increase the clinical suspicion of canine leishmaniasis. Moreover, especially in the early phases of the disease or with atypical presentations, laboratory changes may occur in the absence of obvious abnormalities at physical examination. Therefore, a basic panel is mandatory when canine leishmaniasis is suspected or when a dog with positive result tests for etiological diagnosis needs to be classified as "exposed", "infected" or "sick". Table 1 summarizes the clinicopathological changes that are found in dogs with leishmaniasis (i.e. "sick" dogs).

1) Hematologic abnormalities

Hematological changes in canine leishmaniasis are non specific.[37,38] Neutrophilia, due to the systemic inflammatory response associated with the infectious diseases, may be particularly prominent if ulcerative cutaneous lesions with secondary bacterial infection are present.[37,39,40] Conversely, numerical or morphological changes in the other leukocyte populations are less common, although lymphopenia, lymphocytosis or eosinophilia are occasionally described[39-42] Amastigotes may be rarely documented in circulating leukocytes of infected dogs (0.5% of cases), within neutrophils, lymphocytes and monocytes (figure 1);[41,43] the percentage of infected cells is so low that their search is generally not rewarding. When a systemic disease and blood dissemination is suspected, more sensitive tests such as PCR or quantitative PCR should be preferred to the simple microscopical examination of blood smears (see below). When parasites are found on smears,

however, it is very likely that the parasite burden is high: therefore, although, as stated above, the simple detection of the parasite does not automatically mean that the infected dog is sick, it is more likely that dogs with a high parasite burden (and parasites detected on smears) are sick than simply infected.

The most common hematological changes in leishmaniotic dogs is anemia,[37,38,44,45] that may appear 6 months after exposure to infection.[27] Anemia is usually mild to moderate and has the normocytic normochromic non regenerative pattern[40,44,45] typical of the anemia of inflammatory disease, whose pathogenesis include a depression of metabolic activity of bone marrow, in this case sustained also by the infection of stromal cells by the parasite, and a decreased iron availability for erythropoiesis.[46] However the pathogenesis of anemia in leishmaniotic dogs include additional mechanisms such renal failure leading to reduced erythropoietin synthesis and secondary dismyelopoiesis associated with the production of antibodies against erythroid precursors.[47] Moreover, it is very likely that anemia also has a hemolytic component,[48] likely due to the presence of anti-RBC antibodies; in a few cases, the hemolysis may be prevalent,[49] or associated with a "lupus-like" reaction along with other dermatological or systemic signs or laboratory change, including positive ANA-test[50] or perinuclear antineutrophil cytoplasmic autoantibodies.[51]

Thrombocytopenia is fairly frequent in leishmaniotic dogs without concurrent infections. It is usually mild to moderate but if severe, co-infections with other vector-borne pathogens (e.g. *Ehrlichia canis*, *Anaplasma phagocytophilum* or *A. platys*) or other possible causes of reduced platelet concentration should be suspected. The most likely mechanism responsible for thrombocytopenia in leishmaniasis is a peripheral consumption of circulating platelets. In turn, this mechanism may be due to immune-mediated mechanisms, since the presence of anti-Plt antibodies has been demonstrated in leishmaniotic dogs.[52-54]

Moreover, platelet loss may be associated to hypercoagulability caused by a decreased concentration of anti-thrombin III, as in any other protein losing nephropathy[55] (see below), or to disseminated intravascular coagulation (DIC) that may be occasionally suspected in leishmaniotic

dogs.[56] However, the mechanism of thrombocytopenia in leishmaniotic dogs includes also a decreased production due to the depressed bone marrow activity and to the secondary dysmyelopoiesis cited above. Even in the absence of reduced platelet counts, however, platelet may be hypofunctional in dogs with leishmaniasis[57] although this reduced function is rarely responsible for hemostatic abnormalities, as described below.

One additional hematological test that may be run in dogs with leishmaniasis is the flow-cytometric evaluation of the CD4/CD8 ratio. The rationale for performing this test is that as soon as Th1 responses decreases, thus increasing the susceptibility to the disease and favoring the shift from latent infection to overt disease, the number of CD4+ lymphocytes decreases causing reduction of the CD4/CD8 ratio.[58,59] Therefore, a seropositive or PCR-positive dog with a low CD4/CD8 ratio is more predisposed to develop clinical signs than a similar dog with normal CD4/CD8 ratio. The practical applicability of this test, however, is limited by the high individual variability and by the difficulty to determine a clear cut-off for staging the disease. Hence, this test may be useful for monitoring the post-treatment follow-up, rather than to stage a dog at first diagnosis of leishmaniasis; the authors do not suggest the use of this test for diagnostic purposes in dogs suspected to have leishmaniasis.

Finally, the hematological profile of leishmaniotic dogs may be completed by bone marrow cytology.[27,42,44,60] This analysis may be useful in leishmaniotic dogs to confirm the infection through the detection of infected macrophages, as better specified below, but it may be also used to differentiate a simple infection from systemic disease (i.e. “infected” vs. “sick” dog).[15] Despite some histological studies demonstrated that parasite density can be high despite few clinical signs,[61] generally the parasite load and the presence of cytological alterations increase as soon as the dogs show clinical signs.[62] Therefore, rare infected macrophages may be occasionally seen in the absence of other pathological findings in dogs that are simply infected, whereas dogs in which the parasite induces an inflammatory reaction and/or a systemic disease, are characterized by a higher number of parasites detected cytologically and by a series of morphological changes. Specifically, in the

206 latter case cytology of the bone marrow usually reveals an erythroid hypoplasia,[40] without
207 abnormalities in the ratio between maturative and proliferative pools of erythroid precursors,
208 occasionally associated with myeloid hyperplasia (and thus with an increased M:E ratio). Moreover,
209 signs of bone marrow inflammation, generically defined by Stockham and Scott as "myelitis",[63], are
210 usually found (figure 2). These include a proliferation of either infected or non-infected
211 macrophages often with signs of erythrophagia or, more generically, cytophagia, associated with an
212 increase of neutrophils in the framework of the myeloid hyperplasia, and a moderate to severe
213 plasmocytosis characterized by a higher number of plasma cells with signs of activation (e.g. mott
214 cells), and lymphocytes.[64,65] Megakaryocyte hyperplasia may also be present, especially when
215 peripheral consumption of platelets occurs.
216 Secondary dismyelopoiesis is another common finding, although less frequent than the typical
217 pattern listed above (figure 3). This condition is characterized by multiple peripheral cytopenias
218 (e.g. the anemia and thrombocytopenia cited above) associated with hypercellular bone marrow on
219 which one or more cell lineages show dysplastic features. In canine leishmaniasis, these mostly
220 include dyserythropoiesis and dysmegakariopoiesis, while dismyelopoiesis (and in particular
221 dysgranulopoiesis, mostly characterized by abnormal maturation and ring forms) is only
222 occasionally found: more specifically, erythroid precursors with abnormal mitoses, asynchronous
223 nucleo-cytoplasmic maturation, nuclear fragmentation, and/or late stage maturation arrest,
224 especially when the pathogenesis includes an immune-mediated reaction against RBC precursors.
225 Dwarf megakaryocytes associated or not with an increased number of megakariobkasts and with
226 emperipolesis may be typical aspects of dysmegakariopoiesis in leishmaniotic dogs. The detection
227 of secondary dysmyelopoiesis however, is not per se diagnostic for leishmaniasis, unless
228 macrophages with intracytoplasmic amastigotes and other signs of reactivity (e.g.
229 lymphoplasmocytic infiltration) are found. Therefore, the cause-effect association between
230 secondary dysmyelopoiesis and the simple seropositivity or PCR-positivity should be carefully
231 considered. Ultimately, in this case the diagnosis of leishmaniasis should be based on the exclusion

232 of other infectious, toxic or metabolic causes of secondary dysmyelopoiesis or of primary
233 myelodysplastic syndromes (MDS) as recommended in hematology textbooks.[45,47,64]
234 The last and less common finding in bone marrow cytology leishmaniotic dogs may be the presence
235 of erythroid hyperplasia when peripheral signs are consistent with the diagnosis of immune-
236 mediated hemolytic anemia (IMHA). Also in this case, however, IMHA can be associated with
237 leishmaniasis only if the parasite if found in cytological specimens characterized by erythroid
238 hyperplasia. If not, the diagnostic approach should be taken into account only if any other possible
239 cause of IMHA has been excluded. In brief, bone marrow cytology may be useful for diagnostic
240 purposes in some dogs, by detecting amastigotes and compatible cytological abnormalities, or to
241 differentiate between infected dogs from those that are sick due to leishmaniasis.
242
243 *2) Hemostatic abnormalities*
244 Hemostatic abnormalities are uncommon in leishmaniotic dogs. Activated partial thromboplastin
245 time (aPTT) and prothrombin time (PT) may be increased; however, in most cases this is due to
246 preanalytical factors, since their prolongation may occur when the concentration of total globulins
247 increases, which is frequent in dogs with leihsmaniasis.[66] Alternatively, prolonged coagulation
248 times may result from DIC, although this complication is uncommon in leishmaniotic dogs.[56,67]
249 Conversely, hypercoagulability may be common in leishmaniotic dogs if affected by severe protein
250 losing nephropathy.[68] This is mostly due to glomerular loss of antithrombin III (ATIII), a protease
251 inhibitor involved in the regulation of blood coagulation that prevents the conversion of fibrinogen
252 into fibrin. The lack of this physiologic anticoagulant may induce hypercoagulability that in turn
253 promotes thrombosis and subsequent consumption coagulopathy. Hypercoagulability is also
254 favored by the hyperviscosity syndrome due to increased circulating globulins.[69] As in any other
255 hypercoagulable state associated with protein losing nephropathy, hypercoagulability of
256 leishmaniotic dogs was also demonstrated through a decreased clot formation time and an increased
257 global clot strength using thromboelastography (TEG);[70] differently, in another study the

coagulation profile of leishmaniotic dogs assessed by thromboelastometry (TEM, a technique similar to TEG) was within normal limits.[71] However, it is worth noting that TEM and TEG are affected by the RBC mass, possibly explaining the different results obtained with TEM and TEG.[72,73] In brief, to assess hypercoagulability in dogs with protein losing nephropathy associated with leishmaniasis the authors currently suggest including only ATIII measurement.

3) Biochemical abnormalities

Because the clinical presentation of dogs with leishmaniasis is variable, also the type of biochemical abnormalities varies accordingly. Renal dysfunction and inflammation/immune reactions are frequently observed and their presence should be evaluated in each dog with suspected or confirmed leishmaniasis (see below). Regarding other routine biochemical analytes that may be altered in leishmaniotic dogs, there are enzymes of hepatobiliary or pancreatic damage that may increase in case of pyogranulomatous infiltrates affecting these organs,[15,16] muscular enzymes, including LDH and CK, that may increase with musculoskeletal lesion.[74] Nevertheless, increased CK may also be due to the increased CK-BB isoenzyme if neurological signs are present,[75] since *Leishmania* has been found in the brain of some affected dogs with cerebrovascular alterations,[76-78] or to increased CK-MB if leishmaniotic dogs have cardiomyopathy (i.e. increased tropoinin I and cardiopulmonary lesions have been reported in many leishmaniotic dogs).[79-81] Biochemical abnormalities consistent with alteration of endocrine organs are rare, despite amastigotes associated with inflammatory lesions have been found in the adrenal cortex of leishmaniotic dogs.[82,83]

Assessment of renal function

The systemic immune complex disease that characterizes leishmaniasis induces the deposition of circulating immune complexes at the glomerular level. This induces a series of inflammatory glomerular changes detectable histologically and ultramicroscopically,[36,84,85,86,87] that are primarily responsible for a proteinuric nephropathy.[87] The evolution of this condition, as for any other

284 protein-losing nephropathy, is represented by the development of a chronic kidney disease (CKD)
285 due to a series of factors that include progressive glomerulosclerosis, renal hypertension, overload
286 of protein reabsorption in tubular cells, with subsequent tubulointerstitial nephritis[85,87] In turn,
287 advanced stages of CKD are characterized by hyperazotemia and may be associated with systemic
288 hypertension, both factors contributing to comorbidity in dogs with leishmaniasis.[87,88] Therefore, as
289 for any other renal disease, the early detection of proteinuria and correct classification of CKD is
290 mandatory in the diagnostic workup. To this aim, the clinical and laboratory approach is the same
291 recommended by the International Renal Interest Society (IRIS)[89] for any type of CKD. This
292 approach is based on a thorough clinical evaluation of the dog, based on physical examination and
293 diagnostic imaging, on the measurement of arterial pressure according to the guidelines
294 recommended by the American College of Veterinary Internal Medicine (ACVIM)[90] and on the
295 quantification of urinary proteins (described in the section of this article regarding urinalysis) and
296 markers of renal function. According to the IRIS guidelines, these include mostly the urine specific
297 gravity and the serum concentration of creatinine.[89] This latter has been shown to increase
298 frequently in leishmaniotic dogs[14,15,16,19,20,91] and early (from 12 to 18 months) following infection.[27]
299 Creatinine is a good indicator of a decreased glomerular filtration rate (GFR), and it more specific
300 than other markers such as urea[92,93] but unfortunately they are not enough sensitive to detect the
301 earliest stages of renal insufficiency. Due to the compensatory activity of residual nephrons, in fact,
302 an increase of serum creatinine occur only when the GFR dramatically decreases.[93] Therefore,
303 although creatinine is commonly used to detect overt CKD, a huge research activity is currently
304 running in veterinary nephrology and clinical pathology to identify earlier markers of decreased
305 GFR, either in leishmaniotic dogs or in dogs affected by other types of CKD. The direct
306 measurement of GFR trough clearance tests (e.g. clearance of inulin, exogenous creatinine or, more
307 recently, iohexol) would be the best method to assess in real time the functionality of the
308 kidneys[94,95] Unfortunately these tests, although currently validated for use in dogs, are not widely
309 available in clinical practices or in veterinary laboratories. Therefore, the diagnostic potential of

310 indirect markers other than creatinine has been recently investigated. Cystatin C (Cys C) has been
311 proposed as surrogate marker of creatinine, especially using advanced measurement techniques
312 such as particle-enhanced turbidimetric immunoassay.[96-99] The serum concentration of Cys C has
313 been assessed also in dogs with leishmaniasis.[100] However, there is no evidence that serum Cys C,
314 that is less specific than creatinine, is more sensitive that creatinine in detecting early CKD.[94]
315 Promising results have been obtained by the measurement of urinary Cys C[101] but this seems not to
316 be true in canine leishmaniasis, where urinary Cys C increases only in proteinuric dogs with
317 increased creatinine.[102] Recently, a promising biomarker for early detection of renal dysfunction
318 seems the symmetric dymethilarginine (SDMA), a catabolite of methylated proteins that is mainly
319 excreted by the glomeruli and not reabsorbed by tubuli ,[103,104] that in published report has been
320 measured in serum using chromatographic techniques.[104] Studies in cats, where the early diagnosis
321 of CKD may be more challenging than in dogs, demonstrated that SDMA increases earlier than
322 creatinine,[105,106] although it does not provide additional advantages in animals with overt CKD (i.e.
323 with CKD on which serum creatinine is already increased). Preliminary results indicate that SDMA
324 may have the same role of early indicator of renal dysfunction also in dogs,[104,107] although a few
325 extrarenal variables may affect the results.[108] Therefore, although no studies specifically focused on
326 the use of SDMA in canine leishmaniasis exist, it is very likely that SDMA may be used, in the
327 future, to assess renal function in leishmaniotic dogs that are proteinuric but still have normal
328 creatinine concentration.
329 Other blood markers may provide additional information in leishmaniotic patients with CKD. For
330 example in people the increased serum concentration of homocysteine (Hcy), endothelin-1 (ET-1)
331 or C-reactive protein (CRP) may predict, hypertension and/or inflammation, especially in
332 association with CKD.[109-113] In dogs, Hcy increases in those with CKD[114] but seems to be less
333 associated with hypertension than in humans.[115] Conversely, ET-1 increases in serum of dogs with
334 hypertension associated with early stages of CKD[115] and is also associated with inflammation, as
335 demonstrated by its correlation with CRP levels, which in turn increases mostly in CKD associated

with inflammatory conditions.[113] However, the potential of these additional markers as complementary tests for the management of leishmaniotic dogs with CKD has not been investigated. Conversely, inflammatory markers such as CRP, ferritin and adiponectin have been measured in the urine of dogs with leishmaniasis and were found increased in dogs with proteinuria, sometime in the absence of elevated serum creatinine.[102,116,117] These analytes might therefore work as early markers of CKD in leishmaniotic dogs, although it is more likely that their increase depend on the elevated serum concentration, thus reflecting the inflammatory state typical of leishmaniasis, rather than a direct consequence of CKD.

Finally, in leishmaniotic dogs, tubulointerstitial lesions may occur secondarily to proteinuria caused by glomerular damages. Therefore, the availability of markers to identify dogs with tubular lesions would allow to early differentiate dogs with pure glomerular lesions from dogs with more advanced renal damage. Tubular markers, however, are usually measured in urine and not in serum and are described in the section on urinalysis. It is also worth mentioning that some dogs with CKD may have acute deterioration of their renal dynsfunction due to factors related or not to leishmaniasis (e.g. vomiting, diarrhea).

Assessment of inflammatory/immune reactions

Based on the pathophysiology above described, it is clear that leishmaniotic dogs with overt disease have an intense inflammatory reaction and produces high amount of molecules involved in the immune response, including antibodies. Both these phenomena may be investigated using tests such as protein analysis and serum protein electrophoresis or measurement of acute phase proteins (APPs).

Protein analysis and serum protein electrophoresis are considered the most accurate tests to diagnose canine leishmaniasis. Protein changes appear 12-18 months after exposure to the parasite.[27] Total proteins and total globulin are frequently increased, especially in the acute phase of

362 the disease.[14,15,16,21,91,118,119] The increase of total protein can correlate with the severity of the
363 clinical score, according to Proverbio et al.[120] Moreover albumin decreases both because of its role
364 as negative APPs (see below) and of the renal loss associated with proteinuric nephropathy leading
365 to decreased albumin:globulin (A/G) ratio[91,118,119] The decrease of the A/G ratio is so frequent that it
366 has been considered by some Authors to be one of the more sensitive tests for canine
367 leishmaniasis[118] and hypoalbuminemia is considered a negative prognostic factor in leishmaniotic
368 dogs since the magnitude of hypoalbuminemia negatively correlates with survival times.[121] The
369 typical electrophoretogram of leishmaniotic dogs with overt clinical signs (figure 4) displays
370 hypoalbuminemia, an increase of α_2-globulin, where most of the positive APPs migrate, and
371 especially a strong increase of γ-globulins, due to the huge amount of circulating antibodies,
372 immunecomplexes, and other molecules with γ motility involved in immune-mediated
373 inflammatory reactions.[119] Occasionally, peaks due to circulating antibodies are found in the
374 β region, where IgM and some APPs migrates. The gammopaty is typically polyclonal but
375 sometime the peak may be narrower (oligoclonal), biclonal[122] or definitely monocloncal.[123] The
376 interpretation of the peak must take into account the electrophoretic method, since peaks are usually
377 narrower using capillary zonal electrophoresis (CZE) than using agarose gel or cellulose acetate.[124]
378 Therefore, CZE may lead to falsely interpret as monoclonal a peak that is ultimately oligo- or
379 polyclonal (figure 5). Moreover, although monoclonal peaks associated exclusively with
380 leishmaniasis have been described, the detection of monoclonal peaks should induce to consider the
381 possible presence of concurrent diseases (e.g. other vector-borne diseases or multiple
382 myeloma).[125,126]
383
384 Acute phase proteins are powerful indicators of inflammation: the pro-inflammatory cytokines
385 produced in inflammatory sites induce the so called “acute phase response”, characterized by the
386 release of neutrophils from storage pools, by an activation of myelopoiesis (see above), and by a

387 modulation of protein synthesis in the liver.[119] This latter phenomenon leads to a decreased serum
388 concentration of the "negative APPs", and to an increased concentration of the "positive APPs" that
389 includes a series of immunomodulators, scavenger or transport proteins, antiproteases, and other
390 proteins involved in host defenses.[112] Therefore it is not surprising that the serum concentration of
391 positive APPs in dogs with overt canine leishmaniasis is high. The list of APPs whose
392 concentration increases in serum of leishmaniotic dogs is long and includes CRP, Haptoglobin
393 (Hp), Ceruloplasmin (Cp) Serum Amyloid A (SAA) and ferritin,[127-132] with CRP and ferritin that, as
394 previously reported, increase also in urine. Similarly, a decrease of negative APPs other than
395 albumin has also been reported; these are transferrin, indirectly measured as total iron binding
396 capacity (TIBC), that induces also a reduction in the concentration of iron, and a decreased activity
397 of the enzyme paraoxonase (PON-1).[129,130,133] PON-1 is a negative APP that is bound to high
398 density lipoproteins (HDL) and represents a link between inflammation and oxidative stress.
399 Therefore its decrease is not constantly seen in leishmaniotic dogs but it may become evident when
400 oxidative stress is particularly severe.[133] Interestingly, in these cases also the concentration of HDL,
401 that is converted into low density lipoprotein (LDL) after detachment of PON-1, decreases[134] and
402 may be a cheap marker of inflammation and oxidative stress associated with leishmaniasis.
403 Recently a reduced serum activity of adenosine deaminase (ADA) and butyrylcholinesterase
404 (BChE), two enzymes involved in modulating immune responses, has also been reported in dogs
405 with leishmaniasis.[135]
406 The APP changes summarized above are not diagnostic *per se* since mild increases of positive
407 APPs have been reported also in infected dogs without clinical signs[127] and severely increased
408 levels may occur in diseases other than leishmaniasis.[112] In a dog in which leishmaniasis has been
409 diagnosed by other clinical or laboratory findings, however, the magnitude of these changes may
410 reflect the magnitude of inflammation and thus provide prognostic information. In particular, the
411 decrease of PON-1 is particularly evident in severe diseases and may therefore be a negative
412 prognostic marker. Moreover, APPs may be useful to monitor the follow up.

413

414 *4) Abnormalities at urinalysis*

415 The analysis of urine from dogs with suspected or confirmed leishmaniasis must be focused on
416 three main aspects: confirmation of CKD, evaluation of proteinuria, and investigation of possible
417 tubular damage. Urine samples should be analyzed using the usual approach followed in any case of
418 CKD, that starts with the physico-chemical analysis and continues with sediment analysis and
419 evaluation of proteinuria, eventually followed by the measurement of markers of tubular damages.

420

421 *Physico-chemical analysis*

422 According to standardized procedures,[136] after centrifugation of urine to separate supernatants from
423 the sediment, the routine physicochemical analysis must be addressed to evaluate with a
424 refractometer the urine specific gravity (USG), that tends to decrease in dogs with tubulo-interstitial
425 damage.[93] Then, the supernatant can be used for a dipstick analysis, that should be focused on
426 assessing:

427 - the pH that, although not extremely accurate[137] may be useful to properly interpret other
428 dipstick results: for example the semiquantification of proteins may provide erroneously
429 positive results in alkaline urine (pH>8); [93]
430 - the concentration of proteins, to be interpreted as described below;
431 - the presence of glucose, that, as specified below, may be an indicator of tubular damage.

432 Sediment analysis is another important step of urinalysis of leishmaniotic dogs: the presence of an
433 active sediment (e.g. a sediment with high numbers of leukocytes, erythrocytes or bacteria) is
434 important either because may indicate a lower urinary tract infection superimposed on the primary
435 disease (leishmaniasis) or because in the presence of an active sediment, results regarding
436 proteinuria should be carefully considered;[138] conversely, the presence of granular or cellular casts
437 may be consistent with tubular damage.[93]

438

Evaluation of proteinuria

The evaluation of proteinuria is an essential step, since the presence and magnitude of proteinuria is not only a marker of kidney disease, but also a risk factor for the progression of nephropathy.[139] According to the ACVIM guidelines,[140] proteinuria should be assessed in any dog with diseases potentially inducing this condition, such as leishmaniasis. The ACVIM guidelines recommend to collect urines by cystocentesis, to avoid contamination from the lower urinary tract, however, to improve the possibility to frequently monitor dogs at risk to develop proteinuria, a first evaluation may be done on voided samples, since results recorded with the two methods of collection overlap when the sediment is inactive.[141] Additionally, it may be appropriate to screen the possible presence of proteinuria using a dipstick, since it has been demonstrated that if the dipstick is negative or the dogs is likely non proteinuric according to the IRIS classification[89] and any additional evaluation of proteinuria is therefore not necessary.[142] Conversely, if the dipstick is positive it is very likely that the dog is proteinuric and the protein to creatinine (UPC) ratio must be run to quantify the level of proteinuria. Similarly the UPC ratio must be measured in dogs with a weakly positive dipstick result but with a low USG, in order to understand if the weak positive result really reflect the presence of proteinuria or not. [142] In all the cases above, the UPC ratio must be measured to definitely classify the dog as proteinuric (UPC >0.5), borderline proteinuric (UPC= 0.2-0.5) or non proteinuric (UPC <0.2) according to the IRIS classification, recently revised for the diagnosis of glomerular disease.[89,143] In the interpretation of data, particular attention should be paid on results close to these threshold since results may be affected by analytical variability or by the type of reagent or laboratory procedures used to measure urinary proteins.[144-146] Then, especially if the dogs is borderline proteinuric or proteinuric, quantification of proteinuria must be repeatedly assessed (at least 3 times in 2 weeks) because, according to the ACVIM guidelines,[140] additional investigations or treatments should be performed only if persistent proteinuria is confirmed by repeated urinalysis. Alternatively, the analysis of proteinuria can be done on pooled urine samples, in order to reduce the possible influence of circadian variations in renal protein excretion.[147] Finally, according to the

ACVIM guidelines[140] the origin of urinary protein should be assessed through a renal biopsy. However, according to the recent IRIS guidelines[143] renal biopsy is recommended only in the case of rapid progression of CKD or in dogs not responding to conventional treatments. Alternatively, the origin of proteinuria can be argued on the basis of surrogate methods such as qualitative analysis of urinary proteins (see below). However, in the case of very high UPC ratio, as usually occur in leishmaniotic dogs, proteinuria is mostly of glomerular origin and tubular proteinuria, if present, contributes only partially to the concentration of urinary proteins.

Markers of tubular injury

Although the gold standard method to identify the origin of proteinuria is the histological analysis of renal biopsies, due to its invasiveness this technique is rarely applied in leishmaniotic dogs. However, in order to differentiate the dogs with a tubular component of proteinuria, that are in a more advanced stage of renal disease, urinary markers may be used.[148] Some rough markers such as granular or cellular casts and glycosuria in normoglycemic dogs are very specific indicators of tubular damage, but are not enough sensitive and therefore do not detect dogs with early tubular damage. Additionally, tubular damage so severe to induce glycosuria are rarely observed in leishmaniotic dogs. Early information about the presence of tubular damage may be achieved using sodium dodecylsulphate (SDS) electrophoresis of urinary proteins or measuring the concentration or some urinary analyte or the activity of some urinary enzymes. The SDS denaturates and charges negatively the urinary proteins. Therefore, after migration on polyacrylamide gel (SDS-PAGE) or agarose gel (SDS-AGE), proteins migrate according their molecular mass.[149] This allows to differentiate large proteins, that are of glomerular origin, from small proteins, that are of tubular origin. Results of SDS-PAGE have been shown to well correlate with results of renal biopsies, especially for the identification of glomerular damage or of severe tubule-interstitial damages.[150-151] while SDS-AGE, that is more diffuse in veterinary laboratories because it may be fully automated and is more rapid, cheap and less toxic that SDS-PAGE.[151] However, SDS-AGE has some

491 analytical limitations:[152] it is more susceptible to the concentration of urine thus potentially
492 providing false positive or false negative results in very concentrated or very diluted urine,
493 respectively, and may suffer from pre-analytical artifacts such as bands due to storage artifacts or to
494 proteins originating in the male genital tract.[153,154] However, using SDS-AGE it has been
495 demonstrated that most of the leishmaniotic dogs have a mixed (glomerular and tubular) pattern and
496 only a minority of dogs, likely those with less "chronic" CKD, have a pure glomerular
497 proteinuria.[84,155] Occasionally, some leishmaniotic dogs has low molecular weight proteinuria with
498 no signs of glomerular disease, likely due to a free light chain proteinuria (i.e. a pre-renal
499 proteinuria associated with the intense antibody production) rather than to a tubular damage.[156]
500 Enzymuria is considered a good marker of tubular damage, since the enzymes of interest are usually
501 located in the cytoplasms of tubular cells. Therefore, they may be found in urine when tubular cells
502 are damaged. Several enzymes may be used for this purpose but the two most popular are γ-
503 glutamyl transferase (GGT) and N-acetyl-β-N-glucosaminidase (NAG)[93,157] that must be measured
504 just after sampling since their activity decreases after refrigeration or freezing.[158] Increases of these
505 enzymes, as well as of other enzyme such as alkaline phosphatase (ALP) or β-glucuronidase, have
506 been reported in dog with leishmaniasis[159] and the increase of GGT activity has been showed to
507 correlate with the presence of tubular bands in SDS.[155] On the contrary, no information is available
508 on the utility in leishmaniotic dogs of the measurement of other urinary analytes such as clusterin,
509 kidney injury molecule 1 (Kim-1), neutrophil gelatinase-associated lipocalin (NGAL) or retinol
510 binding protein (RBP), used in pharmacology to assess the presence of renal tubular damages and
511 that have been shown to be potentially useful markers in dogs with CKD.[148,160-162]
512
513 ***Tests for etiological diagnosis that may support or confirm leishmaniasis***
514 Tests for etiological diagnosis are used to identify the presence of the parasite or its components
515 (direct tests) or the host's response to the parasite (indirect tests). As previously mentioned, positive

indirect tests (serology) may or may not indicate a current infection. Conversely, positive direct tests (cytology, histology, immunohistochemistry, PCR, culture and xenodiagnosis) demonstrate that the dog is actually harboring *Leishmania* and it is therefore infected. However, as stated above, the relationship between infection and disease should be based on the evaluation of clinical findings and clinicopathologic tests. The most common tests for etiological diagnosis are described below.

1) Serology

Methods

Apart from some techniques such as Western blotting, that is highly accurate but not available in routine practice, or other methods that have been proposed but are not extensively used, such as latex agglutination or methods based on the detection of antibodies through immunosensors or flow cytometry,[163-166] the most common techniques used to detect antibodies are based on three analytical principles: immunofluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and immunochromatographic test (ICT). ICT is the basis of all-rapid "in clinic" assays, which have a major limitation being that they provide results in a qualitative manner (i.e. presence/absence of specific reactive bands).[167] Several commercial ICT kits are available, which employ single or multiple recombinant *Leishmania* antigens to be used on serum, plasma, whole blood or blood spots dried onto filter paper.[168]Several studies have evaluated their diagnostic performances and, in general, they reach similar conclusions: while specificity is quite acceptable, ICT sensitivity is usually low (in the approximate range of 30-70%).[169] Lowest sensitivities are found in infected dogs without clinical signs, the highest ones in those with overt disease.[170] Therefore, ICT may be used as a first "in clinic" test to complete the laboratory evaluation of clinically suspected dogs and, in case of positivity, serology should be repeated by ELISA or IFAT, which provide quantitative results (see below). However, due to the low sensitivity of the ICT test, a negative result may be falsely negative and therefore, if the clinical suspicion persists, tests with higher sensitivity (i.e. IFAT or ELISA) should be performed, Recently, a ICT kit claiming detection

542 of antibodies developed after natural infection but not those elicited by vaccination with the LiESP-
543 based vaccine, has been proposed as a tool to differentiate vaccinated from infected dogs.[171] The
544 principle of the test is sound, and the first studies reported an elevated sensitivity of this ICT
545 format;[172] however, other studies reported a low sensitivity also for this test.[173]
546 IFAT is recognized as the reference method to perform anti-*Leishmania* serology in dogs,[169,174] as it
547 is very sensitive and also highly specific except in areas endemic for the New World parasite
548 *Trypanosoma cruzi,* that may give false positive results; values approach 100% for both the
549 parameters. ELISA is also very sensitive and specific when a combination of immunodominant,
550 recombinant proteins are used as antigen, whereas it has slightly lower specificity when crude
551 parasite lysates are employed instead.[167,173-175] Compared to IFAT, that is based on the evaluation of
552 promastigote fluorescence at UV microscope and is therefore operator-dependent), ELISA is easier
553 to standardize since results are read by an automated spectrophotometer. Both IFAT and ELISA
554 have the advantage to provide quantitative results that are based, respectively, on the final antibody
555 titer (the last sample dilution providing positive result) and optical density values that can be
556 compared with reference titred samples using conversion factors. Based on the unavoidable
557 variability due to operator-dependent or analytical (antigen stability, antiserum or equipment
558 performances) parameters, precise anti-*Leishmania* antibody titers are not universally available.
559 Hence, a titer is considered "high" if it is 4 fold higher than the threshold value of the laboratory.[15]
560 Similarly, 4 fold variations in titers of sequential samples of the same dog should be expected in
561 seroconversions, or in the outcome of therapy. Hence, sequential samples must be analyzed by the
562 same method in the same laboratory.
563
564 *Interpretation*
565 Serological tests detect and quantify the presence of antibodies in serum or plasma. Antibodies can
566 be found in blood as soon as 1 month after exposure to infected phlebotomines, although the
567 median time for seroconversion is about 5 months in natural conditions and 3 in experimental

568 studies).[176] Therefore is very likely that dogs living in highly endemic regions seroconvert during
569 the sand fly activity period (from late spring to early autumn in temperate zones, all over the year in
570 tropical ones),[9] If the infection is efficiently controlled by the host's immune responses, the
571 antibody titers, when present, tend to remain low and therefore these clinically-healthy dogs are
572 classified as exposed or infected.[15] Conversely, the uncontrolled parasite dissemination is associated
573 with an exaggerated humoral response and therefore antibody titers are high when the disease is
574 evident. This condition is classified as "sick dog" or "severely sick dog" by CLWG classification,
575 and stage III or IV (severe or very severe disease) by Leishvet classification.[14] Furthermore, a direct
576 relationship between the clinical score and antibody titers exists.[120,177] However, dogs with clinical
577 signs (sick) and low-medium antibody titers may also be detected. These have been classified as
578 stage I or II (mild or moderate disease) according to the Leishvet classification.[14]
579 Therefore, quantitative serology should always be performed when, despite strong clinical suspicion
580 of leishmaniasis, lesions approachable by fine needle aspiration are not present or when cytological
581 analysis of lesions (including lymphoid organs and bone marrow) does not reveal the typical pattern
582 associated with leishmaniasis, despite a possible PCR positivity. In this case a high antibody titer is
583 often consistent with the disease, while, if the antibody titer is low, leishmaniasis should be
584 considered only if any other disease potentially responsible of the clinical presentation is ruled
585 out.[14,15]
586
587 *2) PCR*
588 *Methods*
589 As for serology, several molecular methods have been proposed to detect the presence of the
590 parasite DNA in various biological samples. Some of these methods are not commonly used or
591 recently validated, such as the non-amplification assay based on the use of probes labelled with gold
592 nanoparticles[178] or the loop-mediated isothermal amplification (LAMP).[179,180] Conversely,
593 conventional PCR, nested PCR and quantitative (real time) PCR are widely used in routine

practice.[14,15,169,174] PCR sensitivity and specificity varies according to the method and especially according to the target DNA sequence. Most of the PCR tests currently used are targeting multicopy DNA sequences, such as the small subunit ribosomal RNA genes or the kinetoplast DNA minicircles, thus increasing the sensitivity of the test.[181] Compared with conventional and nested PCR, the quantitative PCR techniques offer two main advantages:[182] they are usually run in close systems and are therefore less prone to contamination, and provide information about the copies of DNA that are present in the sample. This latter aspect may be relevant during the follow up to monitor the efficacy of leishmanicidal treatments and therefore it may advisable to use quantitative PCR at first diagnosis (before any treatment), in order to have a baseline value for further analyses during the follow up[182,183] However, it does not seem that quantitative PCR techniques are more sensitive than conventional or nested PCR to diagnose leishmaniasis in dogs.[184] One additional limitation of quantitative PCR is that standardized methods to accurately quantify the copies of DNA may not be offered by some laboratories.

Samples

PCR techniques may be applied virtually on any tissue or biological fluids. Theoretically, it may be superfluous to use molecular tests in affected tissues in which amastigotes have been visualized by cytology or histology (see below). However, cytology and histology are less sensitive than PCR (see below) and therefore, a negative cytological result does not exclude that amastigotes are present. Therefore, when a fine needle aspirate or a tissue biopsy is performed, it may be advisable to use part of the material to prepare cytological or histological specimens and to store the remaining sample as recommended by the laboratory (usually frozen, fixed in 95% ethyl alcohol or in appropriate preservatives) to run PCR in case amastigotes cannot be visualized despite the cytological or histological pattern is highly consistent with leishmaniasis. If needed, PCR may also be performed on cytological material already fixed on glass slides[185] or on formalin fixed and paraffin embedded material.[186,187]

In routine practice PCR is rarely run on injured tissues, for which cytology and histology are preferred. When lesions are not present, or they are not approachable by fine needle aspiration or biopsy (for example when the prevalent clinical presentation is anemia or proteinuric nephropathy), other samples are preferred. Several studies demonstrated that bone marrow and/or lymph nodes and spleen provide the highest sensitivity in detecting *Leishmania* by PCR, especially in sick dogs,[14,188-192] pending that the quality of the sample is adequate, especially for the lymph node. Parasite DNA can be easily found in the skin, including intact tissue especially in the facial region or in the ears. Recent studies demonstrated that conjunctival and, to a lesser extent, oral and nasal swab material is very sensitive for the detection of *Leishmania* DNA and, in addition, can provide positive results earlier than other tissues[188,190,193-196] Buffy coat or whole blood may also be used for conventional or quantitative PCR analysis. Although its sensitivity is lower than that of the other tissues cited above, it can be collected without invasive methods and in the case of positive results provide a diagnostic information in a rapid and cheap way[14,15]

Interpretation

When interpreting PCR results it must be kept in mind the difference between infected and sick dogs. Ultimately, the detection of the parasite's DNA indicates that the dog is infected. The correlation between infection and disease should be based on the presence of clinical signs and laboratory abnormalities. From this perspective, the detection of *Leishmania* DNA in lesions with cytological or histological patterns highly consistent with leishmaniasis (see below) supports the diagnosis of disease and, similarly, a positive PCR in blood or bone marrow of a dog with systemic signs of leishmaniasis can support the diagnosis of disease. Conversely, positive results in dogs without signs clearly referable to leishmaniasis do not support the hypothesis that the infected dog is also affected by clinical leishmaniasis, unless any other possible disease is excluded. For example, a transient PCR-positivity in bone marrow may be found a few months from the natural exposure to sand fly bites, without necessarily meaning that the dogs is infected or sick.[9] Similarly,

646 the PCR positivity in intact skin of a dog living in an endemic area does not necessarily suggest that
647 the presence of parasites in the skin will be followed by parasite dissemination throughout other
648 body tissues.[10-13] Skin positive PCR results may in fact be dependent on the presence of recently-
649 inoculated promastigotes, or of amastigotes recently phagocytosed by resident macrophages that, in
650 "resistant" dogs, may efficiently control the infection at local level.[188,192,197]
651
652 *3) Cytology*
653 *Samples and methods*
654 Fine needle aspiration should be performed in all cases showing cutaneous papular or nodular
655 lesions and/or lymph node enlargement.[15] Ulcerative cutaneous lesions can be sampled by scraping
656 the lesion or using less invasive methods such as imprint smears. Additionally reports describing
657 the presence of amastigotes and associated lesions in nodular masses with atypical localization,
658 such as the tongue,[29,33] the testis,[198-199] and oral or nasal masses[200] have been reported and therefore
659 any nodular lesion in dogs with clinical or laboratory signs potentially consistent with leishmaniasis
660 (e.g. anemia, CKD, alterations of the electrophoretograms, positive serology) should be sampled by
661 fine needle aspiration. Nasal lesions may also be sampled using brush cytology[201] Similarly, when
662 clinical or clinicopathological pattern is consistent with leishmaniasis, the possible presence of
663 *Leishmania* should be investigated also in pathological body fluids such as joint fluids,[25,26]
664 effusions,[41] or cerebrospinal fluid although in this latter sample, cellularity is usually so low that
665 PCR may identify the parasite better than cytology.[77] In all these cases, fluid samples must be
666 collected by centesis, following the procedures routinely recommended for each site.
667 When cutaneous lesions or nodular lesions in other organs, lymph node enlargement, abnormal
668 accumulation of fluids are absent but the clinical suspicion of leishmaniasis is high, the possible
669 presence of parasites should be investigated in organs rich of cells of the monocyte-macrophage
670 system, such as bone marrow, lymph nodes or spleen[14,15,60]
671

Interpretation

Cytology aims to demonstrate the presence of amastigotes that are usually found within the macrophages and, when the parasite burden is high and cell lysis occurs, also on the background (figure 6). The detection of amastigotes may be difficult in cutaneous ulcerative lesions, where the presence of necrosis and cellular debris or of contaminating bacteria may mask the presence of amastigotes. Attention should be paid to misinterpret as amastigotes cellular or granular debris that may be present in these lesions.

Additionally, cytology may allow to detect the typical inflammatory patterns associated with leishmaniasis, that are usually characterized by pyogranulomatous inflammation associated with a moderate to severe lymphoplasmocytic infiltration in skin or nodular lesions with atypical localization (figue 5) and, in lymph nodes, by a reactive hyperplasia of variable severity, characterized by lymphoplasmocytic and macrophagic infiltration, usually associated with numerous neutrophils.[60,202,203] Similarly, cytologic patterns typically associated with leishmaniasis may be found in the bone marrow, as described in details in the section dedicated to hematology. Neutrophils, lymphocytes and macrophages can be found also in body fluids of dogs affected by leishmaniasis. Additionally, the fluids may show the features typical of inflammation such as high cell counts and protein contents and, in joint fluids, decreased viscosity and negative mucin clot test.[25,26,41,77,204,205]

The diagnosis of leishmaniasis is easy when amastigotes are detected in samples that shows the cytologic patterns described above. However, when cytologic patterns consistent with leishmaniasis but no amastigotes are seen, leishmaniasis should not be ruled out, since it is known that the diagnostic sensitivity of cytology is low.[169,174] In these cases, tests that have higher analytical and diagnostic sensitivity, such as PCR, must be run. Alternatively, affected tissues can be biopsied to perform histology and immunohistochemistry, as described below. Conversely, when amastigotes are seen in the absence of cytological abnormalities, or cytology is done on bone marrow, lymph node or spleen, positive results must be interpreted carefully, as systemic signs may be due to

698 diseases other than leishmaniasis.[15] Similarly, a diagnostic workup to confirm or exclude
699 leishmaniasis (i.e. the clinical disease associated with infection) should be run when *Leishmania* is
700 incidentally found in lesions that clearly have a different origin. For example, several reports
701 describe the association between the presence of amastigotes and tumors such as lymphoma,
702 transmissible veneral tumors and other types of neoplasia.[206-211] On a practical standpoint in these
703 cases it is important to understand if the dog is affected by both diseases or affected by a neoplastic
704 disease and simply infected with *Leishmania*.
705
706 4) *Histology*
707 Histology can demonstrate the presence of *Leishmania* in routinely hematoxylin and eosin stained
708 sections when cytology provides parasite-negative results in tissues having a cytological pattern
709 highly consistent with leishmaniasis. Compared with PCR, histology has two main disadvantages: it
710 can be more laborious and time consuming, and the identification of amastigotes may be more
711 difficult than in cytological samples. As for the latter, amastigote presence can be confirmed by
712 immunohistochemistry (figure 7),[36,212] in situ hybridization[213,214] or PCR on formalin-fixed and
713 paraffin embedded samples.[186,187] On the other hand, histology has the advantage to provide
714 additional information on the cytoarchitectural pattern of the lesions. This is a great advantage since
715 it may allow to discriminate dogs in which the parasite is associated with typical lesions from those
716 in which the infection does not seem to be associated with the disease. Therefore, according to some
717 guidelines,[22] histology should always be performed, especially in the case of cutaneous lesions. The
718 interpretation of histological results is facilitated by the elevated number of papers describing the
719 distribution of parasites and the lesions associated with active disease, mostly characterized by
720 lymphoplasmacytic or granulomatous-pyogranulomatous inflammations and/or by vasculitis either
721 in organs usually affected by *Leishmania* (bone marrow, spleen, skin, lymph nodes, kidney, etc) but
722 also in unusual sites such as heart, lung, adrenal gland, genital tract, central nervous system, skeletal

723 muscle, gastrointestinal tract, nails, lacrimal glands and ocular muscles.[23,24,29-31,33,36,74,76-78,80-82,84-
724 86,156,202,215-221]

725

726 *5) Parasite culture and biological test for infectiousness (xenodiagnosis)*

727 Conclusive diagnosis of active infection should be based on tissue cultures, which not only confirm
728 whether dogs harbor parasites, but also demonstrate that the protozoa is viable and multiplies
729 actively. A diagnostic *Leishmania* culture, which differs from maintenance culture of laboratory-
730 adapted strains, requires biphasic blood-agar media that need fresh components.[75] A conclusive test
731 for infectiousness (xenodiagnosis) requires that naive (laboratory-reared) sand flies are induced to
732 feed on infected dogs and are examined thereafter for the presence of promastigotes in the gut.[222]
733 However both tests are unpractical, since on one hand specific blood-agar media are unavailable
734 commercially for diagnostic *Leishmania* culture and must be prepared in laboratory, on the other
735 hand only a few centers breed sand flies for diagnostic purposes. Therefore these tests are mainly
736 intended for research and cannot be recommended for routine practice.

737

738 ***Future perspectives***

739 Several studies investigated the diagnostic potential of innovative markers in dogs with
740 leishmaniasis: for example, the measurement of iron superoxide dismutase (Fe-SODe) secreted by
741 the parasite has been evaluated as a possible marker of infection;[223] proteomic analysis revealed a
742 series of proteins that are over- or under-represented in leishmaniotic dogs;[224] the analysis of the
743 expression of cytokines or molecules such as leptin or inducible nitric oxide synthetase (iNOS) in
744 blood or tissues revealed different profiles in leishmaniotic dogs compared to controls[11,13,225-227]
745 high levels of matrix metalloprotieinases (MMP), and especially of MMP9 and pro-MMP2, have
746 been reported in serum or CSF of leishmaniotic dogs.[228,229] However, none of the studies provided,
747 to date, exhaustive information on the possible utility in practice of these markers. However, the

748 information provided in these investigations is useful to design future research and explore their
749 potential clinical application.
750 Similarly, the attention of researchers has been recently focused on markers of oxidative stress;
751 inflammation is characterized by the release of reactive oxygen metabolites from phagocytes
752 recruited in inflammatory sites and this leads to a consumption of antioxidant compounds.[230]
753 Increases of oxidants or oxidized molecules (e.g. reactive oxygen metabolites, malonyldialdeide,
754 lipoperoxides, thiobarbituric acid reacting substances) and decreases of antioxidant compounds
755 (total antioxidant capacity, trace elements, paraoxonase) have been reported in leishmaniotic
756 dogs[130,133,134,231-234] Unfortunately, only a few studies assessed the diagnostic potential of oxidative
757 markers. for example, the diagnostic performance of ROS were not excellent, likely because
758 oxidative phenomena are intense when inflammation is superimposed to *Leishmania* infection
759 during the clinically overt phase, but depressed if macrophages have been recently infected, due to
760 the ability of the parasite to inhibit the production of oxygen radicals by phagocytes.[7,8,234]
761
762 ***Tests for monitoring the post-treatment follow up***
763 Laboratory tests during the follow up should be focused in monitoring possible toxic effect of
764 treatment as well as the clinical and the parasitological status of the patient following administration
765 of drugs according to conventional treatments protocols. These mainly include the administration of
766 antimonials and allopurinol or of miltefosine and allopurinol. Treatments different from those listed
767 above should be carefully considered only when conventional treatments are not effective.[235]
768
769 *Monitoring the possible toxic effect of treatment*
770 Theoretically, the possible toxic effects of treatment should be monitored. However, despite some
771 studies reported possible nephrotoxicity of antimonials,[85,236] others did not confirm this finding,[237]
772 and recent investigations demonstrated that no toxic effects on heart or pancreas are induced by
773 these drug category in dogs, differently from what is observed in humans.[238,239] Therefore, toxic

774 effects should be monitored only in selected dogs, particularly when peculiar clinical findings are
775 present or history might lead to hypothesize any drug adversity. The only possible adverse effect of
776 allopurinol is the formation of xanthine crystals, and possibly urolithes, in urine.[240] These occur
777 very frequently and may be sometime abundant although associated clinical signs and urolith
778 formation are less common and suspension of treatment is unusual. Therefore, the analysis of urine
779 sediment should be always included in the laboratory workup when allopurinol is included in the
780 treatment protocol or when urine appears macroscopically turbid or forms an evident pellet after
781 centrifugation (figure 8).
782
783 *Monitoring the clinical status*
784 The clinical presentation of leishmaniotic dogs may be extremely variable. Therefore, it is not
785 possible to define, *a priori*, a common and standardized laboratory work up to be used during the
786 follow up. However, two main aspects need to be monitored each time, namely the presence of
787 renal disease and inflammation.
788 Renal function should be evaluated through the analysis of serum concentrations of creatinine and,
789 especially, through sequential quantification of proteinuria, due to its role as a risk factor for the
790 progression of CKD.[139] Proteinuria has been recently shown to be a negative prognostic factor in
791 leishmaniotic dogs.[121] After conventional leishmanicidal treatment, the degree of proteinuria
792 decreases in 4-8 weeks,[241] thus, additional pharmacological treatments for proteinuria should be
793 decided thereafter. The possibility to restore normal renal function depends on the severity of renal
794 damage at the time of first diagnosis. Therefore, creatinine and proteinuria should be repeatedly
795 assessed during the follow up. The frequency of testing depends on the severity of CKD: dogs in
796 IRIS stages 3 or 4[89] should be frequently tested also during the treatment period. Conversely, dogs
797 in IRIS stages 1 or 2[89] should be tested at the end of the first treatment cycle and then every 6-12
798 months.[242] This latter approach is also recommended by the IRIS Glomerular Disease Study
799 Group,[243] that suggests to evaluate renal functions one month after the end of leishmanicidal

800 treatment, and to decide the frequency of further testing depending on the post-treatment IRIS
801 stage: specifically, tests should be run after 12 months in stage I dogs, every 6 months in dogs in
802 stage 2, every 3 months in dogs in stage 3 and avery 6 weeks in dogs in stage 4.
803 The inflammatory status may be monitored through sequential analysis of electrophoretograms and
804 of acute phase proteins, whereas the simple evaluation of total protein, albumin or A/G ratio, may
805 not be helpful because it is very likely that, despite treatment decreases globulin concentrations,
806 albumin concentrations remain low in dogs with persistent glomerular damage and proteinuria, in
807 turn leading to only minor changes in the A/G ratio. Differently, serum protein electrophoresis
808 allow to detect a progressive decrease of α-and γ-globulins. These decreases start to become evident
809 after 2-3 weeks and 4-6 weeks, respectively, following treatment with antimonials.[244] Therefore, the
810 first useful electrophoretogram to monitor the efficiency of treatment should be run not earlier than
811 one month after treatment begin.[242] The complete normalization of electrophoretograms, however,
812 requires at least 90-120 days[240] If after 2-3 months the electrophoretograms still show abnormal
813 profiles, the possible presence of concurrent diseases such as other vector-borne diseases should be
814 considered, especially if the gammopathy tends to be characterized by narrower peaks (see figure
815 4). Treatments with miltefosine or with other drugs may require longer times to be beneficial (more
816 than 2 months to observe a decrease in γ-globulins) and are also characterized by more frequent of
817 relapses after transient normalization of laboratory profiles.[245,246] Compared with serum protein
818 electrophoresis, monitoring the concentration of APPs provides earlier information regarding the
819 success of treatments with antimonials. CRP and SAA start to decrease in two weeks after treatment
820 and may return within the reference intervals in about one month.[131,132,244] The normalization of
821 PON-1 and HDL is even more rapid: significant increases may be observed 3-7 days after treatment
822 and values return within the reference intervals in two weeks.[112,244] Therefore, to assess the efficacy
823 of treatment, it may be advisable to measure the serum activity of PON-1 or the concentration of
824 HDLs or APPs 1-2 weeks after the first administration of drugs, when other clinical or
825 clinicopathological changes are likely still abnormal.

826

827 *Monitoring the parasitological status*

828 As at first diagnosis, the parasitological status can be monitored indirectly, through the assessment
829 of antibody titers, or by direct evaluation of the parasite presence.

830 In case of successful treatment, a decrease of antibody titers may be expected over time; serology
831 can be repeated during the follow up. However, antibody titers tend to decrease at least 6 month
832 after treatment with antimonials combined with continuous allopurinol administration.[240] Hence,
833 serology should not be included in the first laboratory tests performed shortly after treatment to
834 monitor the clinical status. Antibody titers should be determined during subsequent evaluations.[242]
835 With regard to serological results, it should be kept in mind that a complete negativization of
836 antileishmanial antibodies is unlikely, especially for dogs living in endemic areas that may be
837 repeatedly exposed to the parasite, boosting the antibody response. Therefore, sequential serological
838 tests during the follow up should aim to assess whether antibody titers decrease to values consistent
839 with the simple exposure (i.e. to less than 4 folds the threshold value of the laboratory).[15]

840 In order to assess whether treatment completely eradicates the infection, ideally the presence of
841 parasites should be assessed in the tissues in which the parasite may establish a latent infection and
842 using very sensitive techniques. For this purpose the residual parasites burden should be evaluated
843 with repeated quantitative PCR analyses on bone marrow, spleen or lymph nodes, if still palpable.[15]
844 However this procedure is invasive and it is difficult that owners accept the analysis, especially if
845 treatment has been successful and the dog is clinically healthy. Therefore, in routine practice the
846 evaluation of treatment efficacy is usually assessed with serology or quantitative PCR analysis in
847 blood. If treatment is successful, the latter test may show a clear decrease of the *Leishmania* DNA
848 copies after 3 to 6 months of therapy, with complete negativization occurring between 6 and 12
849 months.[182]

850

851 ***Conclusive remarks and recommended protocols***

852 Diagnosing leishmaniasis in dogs may be difficult due to the complex pathogenesis and broad
853 spectrum of clinical and clinicopathological findings. Hence tests that need to be included in the
854 diagnostic protocol vary in each case or epidemiological scenario.[247]
855 In clinically healthy dogs living or having travelled to an endemic area serology may be the test of
856 choice to assess any possible exposure to parasites. Based on the median time to achieve
857 seroconversion,[176] serology should be performed at least 6 months after exposure, or periodically
858 (e.g. every 6 months-1 year) in dogs living in endemic areas. If serology is positive, it is important
859 to quantify the antibody response: a low antibody titer may be consistent with exposure or an early
860 phase of infection, while a high antibody titer may sugges infection or disease.[14,15] Therefore, the
861 subsequent diagnostic steps should confirm the suspected infection through cytological and PCR
862 analysis of sensitive tissues, and/or identification of possible clinical or laboratory alterations,
863 especially in dogs with high antibody titers. If serology or PCR is positive and tests have been
864 performed beyond the transmission season, the laboratory workup should identify the most common
865 abnormalities of dogs with leishmaniasis but no clinical sign (e.g. anemia, abnormal serum protein
866 electrophoresis, proteinuria). If changes are detected, additional clinical or laboratory tests must be
867 performed in order to stage the disease (e.g. tests recommended by the IRIS guidelines for CKD,[89]
868 tests to quantify the acute phase response of inflammation).
869 If the dog is examined because clinical abnormalities are evident, the veterinarian should try to
870 sample any accessible lesion to obtain cytological smears.[15] If amastigotes are documented and the
871 cytological pattern is consistent with leishmaniasis the dog is considered sick; thus, next diagnostic
872 steps should clarify whether a systemic involvement is also present (e.g. hematological disorder,
873 inflammation, nephropathy) and quantify the antibody titer or parasite burden with quantitative
874 PCR to obtain baseline values useful during treatment follow-up. Conversely, if amastigotes are not
875 observed but cytological patterns are consistent with leishmaniasis, the lesion can be further
876 analyzed by histology combined with immunohistochemistry, in situ hybridization or PCR.[14,15] A
877 positive result with one of these additional tests should lead to investigate further the general health

878 status of the sick dog; conversely, if tests are negative, the presence of infection should be assessed
879 in the bone marrow through cytology and/or PCR and, in case of positive results, further
880 clincopathological tests should be performed as above.[14,15] Regarding serology, its use is advisable
881 to confirm the disease in dogs suspected to have leishmaniasis but yielding no parasites at
882 cytological examination. Higher titers are more likely to occur in dogs with leishmaniasis and a
883 more severe clinical presentation.
884
885 **Conflict of interest statement**
886 The authors are members of the Canine Leishmaniasis Working Group (CLWG). None of
887 the authors of this paper has a financial or personal relationship with other people or organisations
888 that could inappropriately influence or bias the content of the paper.
889
890 **Acknowledgements:**
891 The authors are grateful to Dr. Alessia Giordano for the collaboration in revising the manuscript.
892
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